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PRINCIPAL INVESTIGATOR: David B. Powers, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, California 94143-0962

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We have selected high affinity, specific single chain antibodies that recognize two key components of the metastatic phenotype: urokinase-type plasminogen activator (uPA) and its cell surface receptor (uPA-R). A total of six different scFv antibodies were found for each of the two antigens. Competition ELISA assays show that 5 of the 6 anti-uPA-R scFv antibodies compete with uPA for binding to the receptor and therefore are candidates for scFv which may inhibit metastasis by disrupting the uPA:uPA-R interaction. We have also developed the technology to convert a scFv selected from a phage display library, which due to its small size has an extremely short serum half life in mouse, into a longer lived format to allow for in vivo anti-metastasis assays. We have accomplished this by creating plasmid vectors that fuse the scFv to the human IgG1 Fc domain. The resulting scFv-Fc fusion is expressed to high levels in yeast cells, retains the affinity and specificity of the parent scFv, and has a serum half life in mice of 93 hours, similar to a IgG molecule.

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David B Power

PI - Signature Date

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1. INTRODUCTION

1.1 Overview and goals of the project

For the breast cancer patient, it is metastasis which all too often ultimately proves fatal. Metastasis is a complex, multistep process by which tumor cells invade surrounding host tissue, enter the bloodstream, travel through the circulatory system to a distant site, and exit from the blood vessel to establish a metastatic colony. The metastatic process involves multiple tumor:host interactions, such as proteolysis of the extracellular matrix and attachment to target tissues; as well as autocrine responses of the tumor cells to motility factors, and angiogenesis-inducing factors. Each step in the metastatic process represents a possible point of therapeutic intervention; and since most of these steps are mediated by interactions involving extracellular molecules, they are in principle amenable to inhibition by engineered antibodies. We will create single-chain human Fv antibody fragments (scFvs) to proteins involved in metastasis by selection from phage display libraries. These scFvs will be used to dissect the contribution of different molecular interactions to the overall metastatic process using various *in vitro* and *in vivo* assays, and may eventually serve as the basis for anti-metastasis therapeutic agents.

As a starting point toward this larger goal, we have created high-affinity scFv molecules directed towards the cell-surface receptor of urokinase-type plasminogen activator: uPA-R. Urokinase (uPA) and its receptor (uPA-R) are key components of the metastatic machinery. Urokinase (uPA) is a 52,000 dalton serine protease which is overexpressed in many breast tumors where it is found in a membrane-bound form through interaction with uPA-R. Substantial evidence exists for a key role of receptor-bound uPA activity in metastasis via proteolysis of basement membranes by activating various zymogens to active proteases. In breast cancer, uPA levels are a prognostic factor for relapse-free and overall- survival. Inhibition of receptor-bound uPA activity, either by blocking uPA protease activity itself or by blocking uPA binding to its receptor can inhibit metastasis in a number of *in vitro* and *in vivo* metastasis model systems. To study the role of the uPA:uPA-R interaction in metastasis, we have prepared high-affinity scFv species to the uPA receptor which compete with uPA for binding. These molecules will be tested for antimetastasis activity using an *in vitro* basement membrane degradation assay and a sensitive *in vivo* metastasis assay using breast tumor xenografts in nude mice.

A key subgoal of this research is to develop the technology to create single chain antibody variants with altered pharmacokinetic properties such that they can be used for *in vivo* assays. This is important not only for the purpose of this anti-metastasis research, but as a key technology to support other research efforts in the laboratory of Dr. James Marks to develop and test antibodies that attack breast cancer through growth factor receptors or molecules involved in angiogenesis. This report will detail results in the selection of single chain antibody fragments (scFv) from phage display libraries toward urokinase and its receptor, and the development of

technology to modify these scFv such that they can be tested for anti-metastasis activity *in vivo*.

1.2 Role of urokinase and its receptor in metastasis

The past two decades of metastasis research have shown the metastatic process not to be a random diffusion of cancer cells throughout the patient's body, but rather a complex, multi-step process by which the tumor cells recruit normal physiological processes involved in cell motility and tissue reorganization for a pathological end. Metastasis involves the following steps: primary tumor growth and vascularization; invasion of surrounding host tissue; passage of tumor cells into the blood or lymphatic system; travel to a distant capillary bed; attachment to endothelial cells or exposed basement membrane; degradation of the basement membrane and tumor cell invasion of the underlying host tissue; and finally growth and vascularization of the metastatic colony, which itself can give rise to further metastases (1).

A key step in this process is tumor cell penetration of the basement membrane underlying endothelial cells which line blood vessels. The basement membrane is a specialized type of extracellular matrix, composed primarily of type IV collagen, laminin, fibronectin, and heparin sulfate proteoglycan, which poses a substantial barrier to tumor cell invasion(2, 3). Tumor cells in the blood must somehow degrade this barrier to penetrate to the underlying tissues for successful establishment of a metastatic colony. A 'protease cascade' involving metalloproteinases (primarily type IV collagenase), the cysteine proteinase cathepsin B, and the serine proteases plasmin and urokinase-type plasminogen activator (uPA) is believed to be responsible for basement membrane degradation(4).

This proposal focuses on the role of uPA and its receptor in basement membrane degradation and tumor cell invasion. Urokinase-type plasminogen activator is a 52,000 molecular weight serine protease, capable of proteolytic cleavage of a key bond in the zymogen plasminogen to create active plasmin. Urokinase has a C-terminal protease domain, as well as amino terminal growth factor and kringle domains. The presence of a cell-surface receptor for uPA was discovered by Vaselli et al. on lymphocyte and monocyte cell lines; the receptor has a K_d (dissociation constant) for uPA of 0.1 to 1.0 nM(5). The receptor has been cloned (6) and shown to be a single-chain glycopeptide of 55 to 60 thousand daltons; consisting of three repeated domains of ~90 amino acids in length. The uPA-R is attached to the plasma membrane through the C-terminus which is in a glycosylphosphatidylinositol (GPI) link (7). Binding of uPA to its receptor has been shown to be mediated by sequences in the amino terminus of uPA (the amino terminal fragment, or ATF) (8), specifically by sequences in the growth factor domain (9).

The importance of uPA and its receptor in metastasis is well known, has been reviewed recently (10) and will be briefly outlined. Urokinase-type plasminogen

activator and its receptor are overexpressed in numerous breast tumors and breast tumor-derived cell lines (11, 12). Levels of uPA-R correlate with worse prognosis in colorectal cancer (13) and squamous cell lung cancer(14). uPA-R is expressed in invasive breast cancer but not in normal breast tissue(15). Both uPA and uPA-R correlate with a worse prognosis (decreased survival times and disease-free interval) in breast cancer patients(16, 17, 18, 19, 20, 21, 22).

The importance of uPA and uPA-R is due to its role in basement membrane proteolysis. uPA initiates the degradation of basement membranes by generation of plasmin which then activates procollagenase IV to an active collagenase(23). The role of uPA in basement membrane degradation was first shown using an invasiveness assay using chorioallantoic membrane system(24). Antibodies to uPA-R inhibit invasiveness of two human cancer cell lines (glioblastoma and melanoma) in an *in vitro* assay using artificial basement membranes(25, 26). Similarly, *in vitro* extracellular matrix degradation by the metastatic human fibrosarcoma cell line HT-1080 can be inhibited by the urokinase inhibitor protease nexin I(27). *In vivo* metastasis of the B16 melanoma line in mouse can be inhibited by pre-incubation with anti-uPA antibodies prior to injection(28). Inhibition of metastasis was shown for human PC3 prostate carcinoma cells in nude mice by either 1) displacement from receptor by an inactive mutant of uPA, or by anti-uPA monoclonal antibodies (29). Expression of recombinant human uPA in H-ras transformed NIH 3T3 cells enhances their ability to form metastases *in vivo* (30). In addition, receptor-bound uPA is believed to be involved both in physiological(31) as well as tumor associated angiogenesis(32).

1.3 Phage display antibody technology

Most previous studies of the role of receptor-bound uPA in metastasis were performed by inhibition with murine monoclonal antibodies. Although highly specific and useful reagents, mouse monoclonal antibodies suffer from a number of limitations. They are time consuming to produce, generally requiring two rounds of immunization before hybridoma construction can even begin. Also mouse antibodies are immunogenic in humans, making them of limited use as human therapeutics. Mouse antibodies are difficult to produce against self (mouse) antigens, or for human antigens which are highly conserved with their mouse counterparts. The large size of intact monoclonal antibodies (~150,000) results in poor tumor penetration for use in cancer immunotherapy against solid tumors. Finally there seems to be a limit in the affinity of murine monoclonal antibodies, with affinities less than 10^{-9} M rarely observed; these affinities may be inadequate for many applications.

These above limitations can be overcome by selecting human single-chain antibody Fv fragments (scFvs) from phage display libraries(33). The ability to express antibody fragments on the surface of viruses which infect bacteria (bacteriophages or

phage) make it possible to isolate a single binding antibody fragment from a library of greater than 10^{10} nonbinding clones. To express antibody fragments on the surface of phage (phage display), an antibody fragment gene is inserted into the gene encoding a surface protein (pIII) and the antibody fragment-pIII fusion protein is displayed on the phage surface. Since the antibody fragments on the phage surface are functional, phage bearing antigen binding antibody fragments can be separated from non-binding phage by antigen affinity chromatography. Depending on the affinity of the antibody fragment, enrichment factors of 20 fold - 1,000,000 fold are obtained for a single round of affinity selection. By infecting bacteria with the eluted phage, moreover, more phage can be grown and subjected to another round of selection. In this way, an enrichment of 1000 fold in one round can become 1,000,000 in two rounds of selection. Thus even when enrichments are low, multiple rounds of affinity selection can lead to the isolation of rare phage. Since selection of the phage antibody library on antigen results on enrichment, the majority of clones bind antigen after four rounds of selection. Thus only a small number of clones need to be analyzed for binding to antigen. Analysis for binding is simplified by including an amber (stop) codon between the antibody fragment gene and gene III. The amber codon makes it possible to easily switch between displayed and soluble (native) antibody fragment simply by changing the host bacterial strain.

Human libraries can be produced without prior immunization by displaying very large and diverse V-gene repertoires on phage. Because the library is of human origin the scFvs are less immunogenic in humans, and since scFvs are roughly 1/6th the size of intact IgG they have superior tumor penetration. Such libraries typically yield scFv with affinities in the nanomolar range; *in vitro* affinity maturation via mutagenesis and 'chain shuffling' can easily and routinely increase affinities to the 10 picomolar range (34). The technique is rapid - a series of four selections to generate initial binding phage for characterization can be done in two weeks. Finally, DNA encoding the scFv is readily available from the phage clone, which facilitates engineering the scFv for increased affinity or avidity, or to add a functionalization domain for use in diagnosis, imaging, or immunotherapy.

We have recently produced a very large (7.0×10^9) human scFv phage antibody library in the vector pHEN-1 using V genes rearranged in vivo (35) In a test of this library, scFv against 11 antigens were generated with a 100% success rate. For each antigen, from 6 to 15 unique scFvs were isolated. Those tested had dissociation constants from 1.2×10^{-8} to 8.0×10^{-9} M, similar to the affinities of antibodies from the secondary murine immune response. Thus we are in a position to rapidly generate multiple scFv species to any antigen, with nanomolar affinity, which can then be affinity matured to picomolar affinities if desired.

1.4 Engineering scFv fragments for use in *in vivo* assays

Antibody and antibody fragments generated in this work will be characterized by a combination of *in vitro* and *in vivo* assays. *In vitro* assays for the metastatic potential of tumor cell lines are based on the cells ability to penetrate a reconstituted basement membrane preparation (Matrigel)(36). Tumor cell penetration through reconstituted basement membranes correlates well with metastatic potential of the cell lines *in vivo* (37, 38, 39). *In vivo* approaches for determining the metastatic potential of human cancer lines are based on injection of the cells into nude mice, which support the growth of a primary tumor. The animals are sacrificed at some time post-implantation of the primary tumor, and metastases to brain, lung, liver, and bone are counted. Human breast cancer cell lines have been traditionally difficult to get to form metastases in these models, making the *in vivo* study of human breast cancer metastasis difficult. However recent improvements by orthotopic injection, and injection of the breast cancer cells along with reconstituted basement membrane (Matrigel) have overcome many of these limitations (40, 41). Additional sensitivity has come from stably transfecting the cancer cell line with a reporter gene (chloramphenicol acetyl transferase or beta galactosidase) which allows the detection of as few as several hundred metastasized cells in distal organs (29, 40, 41).

For a biological effect to be observed in these *in vivo* assays, it is necessary to have a therapeutic molecule that will be sufficiently stable to result in a pharmacologically effective dose over the length of the assay. The *in vivo* metastasis assays described take several weeks to perform. However, the small size of scFvs place them well below the renal threshold for clearance - which means that the half life ($t_{1/2}\ \beta$) of a scFv in a mouse is only a few hours(42), clearly insufficient for this purpose. We therefore require a way of modifying the *in vivo* clearance profile of a scFv, without altering its affinity or specificity, in order to do *in vivo* metastasis assays. This modification should also be rapid and easy to perform to allow for the *in vivo* characterization of numerous different scFv species.

One way to modify a scFv for prolonged serum half life is to reverse engineer the scFv into a full-length IgG format. IgG species generally have half lives in mice of several days to weeks. However, methods for making scFv from phage display libraries into full length IgGs are labor intensive, involving multiple cloning steps for the heavy and light chain variable regions, and also require the time-consuming expression of these species in mammalian cell tissue culture.

We have established a rapid and simple method to render a scFv into a form suitable for *in vivo* metastasis assays. We did this by appending the Fc constant portions of an IgG onto the scFv, creating a scFv-Fc fusion, and expressing the construct in yeast cells (Figure 2). This scFv-Fc fusion retains the original affinity and specificity of the parent scFv while having pharmacokinetics similar to an intact IgG. We thus now have the tools in hand to rapidly render a scFv from a the form

in which it selected from a phage display library into a form suitable for *in vivo* metastasis assays. As a bonus, this scFv-Fc fusion/yeast expression technology is playing a key role in the overall focus of Dr. James Marks' laboratory on the design and testing of other therapeutic anti-breast cancer antibodies targeted towards growth factor receptors and factors involved in angiogenesis.

2. BODY OF REPORT

2.1 Selections on urokinase (uPA) and urokinase receptor (uPA-R)

To identify high affinity single chain antibodies to urokinase (uPA) and its receptor (uPA-R), selections were performed using a large (7×10^9 clones) non-immune human scFv antibody library expressed on the surface of bacteriophage (35). Urokinase was obtained commercially (ICN Biomedical, Aurora, Ohio); soluble urokinase receptor was a gift of Dr. Mark Shuman, University of California at San Francisco. The antigens were coated on the surface of immunotubes (Nunc) at 50 ug/ml in 100 mM carbonate buffer pH 6.2. The tubes containing the immobilized antigens were blocked with 4% powdered milk in phosphate buffered saline (MPBS), then 5×10^{12} phagemid particles were added and allowed to bind for 1 hour. Non-binding phage were washed away (20 washes with PBS + .05 % Tween 20 (TPBS) and 20 washes with phosphate buffered saline (PBS)), then binding phage were eluted with 1.0 ml of 100 mM triethylamine, and immediately neutralized by the addition of 0.5 ml 1 M Tris pH 7.5. The eluted phage were amplified by infection of *E. coli* TG1 cells, and used for the next round of selection. A total of 3 rounds of selection were performed for each of the two antigens.

Selections were monitored by determining the titer of eluted phage at each round of selection; in a typical successful selection the output phage titer will increase in subsequent rounds as phage bearing a scFv that binds to the target antigen are enriched in the phagemid population. Results are shown in Table 1. For both antigens, starting titers of ~10^3-10^4 increased two logs or more to ~10^7 in the final round, indicating successful enrichment of phage displaying scFv specific for the target antigen. At each round of selection, 96 separate clones were picked and grown in a 96 well microtiter plate for analysis.

2.2 Characterization of selected clones

2.2.1 ELISA results

To determine which of the selected phagemid clones were specific for the target antigens, small scale expression cultures of the clones were grown to express soluble scFv fragments for analysis. The library clones are in vector pHEN1 which fuses the scFv clone to the minor phage coat protein p3 through an amber stop

codon (UAG). In suppresser strains of *E. coli* such as TG1 the amber codon is suppressed, resulting in incorporation of an amino acid at the amber codon and fusion of the scFv to the p3 protein. However the suppression is not 100% efficient; more than half of the scFv produced terminate at the amber codon and are released in a soluble form to the culture medium. Thus small scale cultures of scFv phagemid clones in *E. coli* TG1 strain produce sufficient quantities of soluble scFv for ELISA analysis. In addition, the scFv produced from vector pHEN-1 are fused to a myc epitope tag, allowing for detection with anti-myc antibodies.

Expression of scFv for ELISA analysis was achieved as follows: cultures of 96 clones apiece from the first, second, and third rounds of selection of the two antigens were grown by replicating plating from the master plates into fresh 96 well microtiter plates containing 150 ul per well of 2xYT media supplemented with ampicillin and 0.1% glucose. The expression plates were grown at 37° C for 3-4 hours with 200 rpm shaking, until the OD_{550} of the bacteria was approximately 1.0. Then 50 ul of a 4 mM IPTG solution was added to induce scFv synthesis, and the plates incubated for an additional 8-12 hours at 25° C with shaking. Finally the cells were removed by centrifugation, and the scFv-containing supernatants from each well were removed and stored at 4°C for ELISA and other analyses.

ELISAS were performed as follows: 96-well microtiter plates were coated with antigens at 10 ug/ml in 100 mM carbonate buffer pH 6.2 overnight. The next day, the plates were blocked by the addition of 200 ul/well of MPBS (PBS plus 4% powdered milk) for 1 hour. The plates were washed with PBS, then 50 ul of the *E. coli* expression supernatants were added per well and allowed to bind for 1.5 hours at room temperature. Finally after washing, bound scFv were detected with the anti-myc monoclonal antibody 9E10 (Santa Cruz Biochemicals) and an anti-mouse IgG HRP (horse radish peroxidase) conjugate and developed with the peroxidase substrate 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma). Positive clones were defined as those which had an A_{405} reading of at least twice background.

ELISA results are shown in Table 2. For both antigens, the percentage of positive clones from the first to second and from second to third rounds, indicating enrichment of clones bearing scFv specific for the target antigen as desired. No specific binders were detected for 96 random clones from the unselected library for either antigen (data not shown). A total of 37 total clones were detected for urokinase, and 46 for the urokinase receptor.

2.2.2 Sequence Analysis

The positive clones for uPA and uPA-R were analyzed by PCR-BstN1 fingerprinting (33) and finally clones having unique PCR patterns were sequenced by dideoxy chain termination on a Licor model 400L DNA sequencer (data not shown). Sequence analysis was necessary both to determine the number of unique clones for further characterization as well as to design PCR primers for further

subcloning manipulations of these clones (see section 2.3) A total of 6 unique antigen-positive clones were detected for urokinase receptor: the clones identified that bound to uPA-R were denoted G12, A4, C2, F5, B5, and A9. Sequence analysis showed that the selection resulted in also six unique antibody clones to urokinase itself.

2.2.3 Competition ELISA analysis of anti-uPA-R scFv antibodies

scFv antibodies selected on the urokinase plasminogen activator receptor uPA-R were next assayed by a competition ELISA in which we sought to determine if the epitope recognized by the scFv overlapped with the uPA binding site on the receptor. Basically, an ELISA was performed in which uPA-R was bound to an immunoplate and scFv binding to the uPA-R were detected by a sandwich assay as described in section 2.2.1. A second set of parallel ELISA reactions were done in which the scFv binding was done in the presence of 5 ug uPA in 50 ul of PBS to compete with the scFv for binding to the uPA-R. Results were calculated as a percentage of maximum signal seen in the presence of uPA and are shown in Figure 1. The results show that only one out of the six anti-uPA-R scFv (B5) recognizes an epitope that does not overlap with the uPA binding site, as this scFv binds to uPA-R as well in the presence of uPA as by itself. For the other five scFv, significant reduction of maximal signal (30-50%) in the presence of uPA is seen, indicating that the epitopes recognized by these clones at least partially overlap with the uPA binding site on uPA-R. These 5 clones are candidates for antibody scFv fragments that may inhibit metastasis by inhibition of the uPA:uPA-R interaction

2.3 Construction of scFv derivatives for *in vivo* studies

Before the anti-uPA receptor antibodies can tested for anti-metastasis activity *in vivo*, they need to be converted to a format that would preserve the affinity and specificity of the parent scFv, while giving the scFv the long serum half life of an IgG species. This was done by developing the technology to express a genetic fusion of a scFv with the human Fc antibody domains as detailed in the following section. The basic design of this dimeric scFv-Fc fusion is shown schematically in Figure 2. The development of this technology, in addition to its use to further the metastasis studies outlined, is also finding a key role in the laboratory of Dr. Marks for *in vivo* testing of antibodies to growth factors and angiogenesis factors which may be targets for antibody-based breast cancer therapeutics. Appreciation of the importance of this technical objective (designated 3.11 in the original research proposal) to this work and to the overall anti-breast cancer focus of Dr. Marks' lab has led to its acceleration from months 24-30 in the original proposal to months 1-12 in the present report.

2.3.1 Rationale and introduction

The modular nature of antibody molecules allows an almost unlimited number of domain re arrangements. Antibody engineering allows the researcher to design and use a variety of binding domains, effector domains, as well as non-antibody fusion partners (43). One of the more useful antibody fragments is the single-chain Fv, or scFv (44, 45), in which the antibody V_H and V_L domains are joined by a short peptide linker. This monovalent, minimal binding fragment is favored for antibody phage display techniques and phage antibody library construction (33, 46).

Once a scFv has been isolated from a phage display library, the binding domain can be characterized as to affinity, epitope and biological activity, as well as subjected to further affinity maturation. The scFv can be used as is, or engineered into other forms (Fv, Fab, Fab2', IgG, or fused to other proteins).

For some applications the scFv itself is the desired format. The small size of the scFv and its rapid clearance from the blood makes it the molecule of choice for tumor targeting approaches(47, 48). The single-chain nature of the scFv is best-suited for intracellular immunization (intrabody) studies (49). However for other uses, it would be desirable to transfer the antigen-binding properties of the scFv into a full length IgG, to take advantage of avidity effects, effector functions, and the prolonged serum half life of an immunoglobulin. Increasing the serum half life of scFv is particularly important for demonstrating in vivo antigen neutralization since the $t_{1/2}\beta$ of scFv is only 2.5 hours in mice (42).

One approach is to directly engineer a phage displayed scFv into a full length IgG and express it in mammalian cells(50). However this can be a laborious, and the yields from tissue culture can be modest. An alternate method would be to engineer the scFv into a more 'IgG-like' structure. We have constructed a vector to rapidly express an scFv-Fc fusion (wherein the scFv is fused to the hinge, C_H2, and C_H3 domains of human IgG1, see Figure 2) in the methylotrophic yeast *Pichia pastoris.* The scFv-Fc fusion can easily be constructed in a single cloning step from a scFv and is secreted at high levels as a glycosylated dimer from *Pichia* cultures. The scFv-Fc fusion retains the affinity and specificity of the parent scFv, combined with the bivalency, prolonged serum half-life, and Fc-mediated effector function (ADCC) of an IgG.

2.3.2 Materials and methods

Cells and media

Pichia pastoris strain GS115 was obtained from Invitrogen Inc. (Carlsbad, CA); media (YPD, YPDS, BMMY, and BMGY) were prepared according to manufacturer's instructions (Invitrogen, Inc.)

pPIgG1, pPIgG1-C25, and pPIgG1-C6.5 plasmid construction

The Fc fragment (hinge, C_H2 and C_H3) of human IgG1 was amplified from the baculovirus expression plasmid pBHuCγ1 (51) using primers 'HuIgGNotIBack' (5'- AAGGAAAAA GCG GCC GCA GAG CCC AAA TCT TGT GAC AAA-3') and 'HuIgGXbaIFor' (5'- ACGC TCT AGA TCA TTT ACC CGG AGA CAG GGA C-3'), which append *Not*I and *Xba*I sites (underlined) onto the 5' and 3' ends of the PCR fragment, respectively. The PCR fragment was digested with *Not*I and *Xba*I and subcloned into *Not*I-*Xba*I digested pPICZαA (Invitrogen) to generate plasmid pPIgG1 (for "Pichia IgG plasmid 1").

C25, a scFv which binds the botulinum neurotoxin type A binding domain (BoNT/A Hc) (52) was PCR amplified from vector pUC119MycHis-C25 using primers 'C25PichiaBack' (5'- CGGCAG CTCGAG AAA AGA GAG GCT GAA GCT CAG GTC CAG CTG CAG GAG TCT GGG -3') and 'LMB2' (5'- GTA AAA CGA CGG CCA GT -3'). The PCR fragment was then digested with *Xho*I and *Not*I and subcloned into *Xho*I and *Not*I digested plasmid pPIgG1 generating plasmid pPIgG1-C25. Similarly, C6.5, a scFv which recognizes the extracellular domain of the c-erbB-2 oncoprotein(53) was subcloned into pPIgG1 using primers 'C6.5PichiaBack' (5'- CGGCAG CTCGAG AAA AGA GAG GCT GAA GCT GGC CAG GTG CAG CTG GTG CAG -3') and 'LMB2' to create plasmid pPIgG1-C6.5. Following construction, the coding regions for both the constant region sequences and the scFv were resequenced to exclude any PCR-induced errors.

Pichia pastoris electroporation

Transformation of *Pichia pastoris* strain GS115 was achieved by electroporation. 10 ug of plasmid was linearized with *Pme*I, phenol-chloroform extracted, ethanol precipitated, and dissolved in 10 ul of dH_2O. Preparation of electrocompetent Pichia strain GS115 was done as per manufacturer's instructions (Invitrogen, Inc.) 80 ul of electrocompetent cells were mixed with 3-5 ug of linearized plasmid in a 0.2 cm electroporation cuvette, incubated on ice ~5 minutes, and electroporated in a Biorad GenePulser with settings of 1500 volts, 25 uF capacitance, and 400 ohms resistance. After pulsing, 1.0 ml of 4° C 1 M sorbitol was added immediately to the cuvette, and the cells transferred to a sterile 15 ml culture tube. The tube was incubated at 30° C without shaking for 1 hour, then 1.0 ml YPD medium was added to the tube, and the cells were allowed to recover for 2 hours at 30° C at 250 RPM. Transformants were plated (200 ul) on YPDS plates containing 100 ug/ml Zeocin and grown at 30° C to isolate Zeocin-resistant transformants. We found that efficiency of transformation was dependent on the site of linearization, 2-10 -fold more colonies were obtained when we linearized using the *Pme*I site rather than the *Bst*XI site.

Small scale expression and screening of Pichia transformants

Zeocin-resistant transformants of the pPIgG1-C25, pPIgG1-C6.5 and pPICZαA (control) plasmids were grown overnight in BMGY medium at 30° C and 250 rpm

shaking in 100 ml glass culture tubes. The next day, the cells were recovered by centrifugation and re suspended to an OD_{600} of 1.0 in BMMY media to induce, and grown again at 30° C and 250 rpm. Fresh methanol was added to a total of 0.5% to maintain induction at 24, 48 and 72 hours post induction, and samples were taken at 24, 48, and 72 hours. Samples were analyzed by SDS-PAGE followed by silver stain (Biorad Silver Stain Plus) and Western blot with polyclonal goat anti-human IgG (Fc-specific) conjugated to horse radish peroxidase (Sigma A-0170). Detection was with ECL (Amersham).

Large scale expression and purification of scFv-Fc fusions

250 ml cultures of GS115/pPIgG1-C25 (clone 9.20) and GS115/pPIgG1-C6.5 (clone 5.20) were grown overnight in BMGY plus Zeocin (50 ug/ml) and kanamycin (50 ug/ml) until the OD_{600} was 4-6. The cells were recovered by centrifugation, then diluted to an OD_{600} of 1.0 in 1000 mls of fresh BMMY (also containing 50 ug/ml kanamycin) media to induce. The 1000 mls were divided into 4 x 2 liter baffle-bottom flasks, each containing 250 mls of culture to ensure adequate aeration, and grown at 30° C and 250 rpm. Fresh methanol was added to 0.5 % to maintain induction at 24, 48, and 72 hours. After 72 hours, the cells were removed by centrifugation and scFv-Fc fusion protein purified from the supernatant. A protease inhibitor cocktail (25 mls of Sigma product P2714) was added to reduce proteolysis, and the pH of the supernatant was adjusted to 8.0 by the addition of 1/10 volume of 1.0 M Tris pH 8.0. Proteins were precipitated by the addition of 400 grams ammonium sulfate in the cold with constant stirring, over a period of ~2 hours. The precipitate was recovered by centrifugation, dissolved in 40 mls of 25 mM Tris pH 8.0, and dialyzed overnight against two changes of 4 liters of 25 mM Tris pH 8.0 at 4° C. After dialysis, the sample was applied to a 1.5 ml Protein G column (Sigma) that had been previously equilibrated with 25 mM Tris pH 8.0. The column was washed once with 10 mls of 100 mM Tris pH 8.0, once with 10 mls of 10 mM Tris pH 8.0 and eluted with 20 mls of 100 mM glycine pH 3.0. 1.0 ml fractions were collected in Eppendorf tubes containing 100 ul of 1.0 M Tris pH 8.0 to neutralize. Peak fractions were determined by absorbance at 280 nm, pooled, concentrated to 0.5 mls on a Centricon 10 concentrator (Amicon) and finally gel filtered on a Superdex S-200 column (Pharmacia) at a flow rate of 0.5 ml per minute in Phosphate Buffered Saline (PBS). Protein concentration was determined based on absorbance at 280 nM using molar extinction coefficients calculated by the method of Gill and von Hippel (54). We derived a factor of 1.0 A_{280} is equal to 540 ug/ml for C6.5-Fc and 620 ug/ml for C25-Fc, based on a calculated molecular weight of 106,000 for each.

Glycosylation analysis

Samples of the scFv-Fc fusions were de-glycosylated using PNGase F (New England Biolabs) under denaturing according to the manufacturer's instructions and analyzed by SDS-PAGE followed by silver staining (Biorad Silver Stain Plus).

Radiolabelling

The C6.5 -Fc was labeled with iodine-125 using iodobeads (# 28665X, Pierce; Rockford IL) according to the methods described by the manufacturer. Briefly, 0.5 mg of C6.5-Fc in 250 mL 0.1 M Phosphate Buffer (pH 6.5) were combined with 0.5 mCi (1.25 mL) of iodine-125 (#NEZ033H, DuPont NEN, Wilmington, DE 19898), and one iodobead. The mixture was incubated for three minutes at room temperature. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method(55). The immunoreactivity of the C6.5-Fc was evaluated in a live cell binding assay utilizing HER2/*neu* expressing SK-OV-3 cells (#HTB77, American Type Culture Collection, Rockville, MD)(56). Ten nanograms of labeled C6.5-Fc in 100 mL PBS was added in triplicate to 5 x 10^6 SK-OV-3 cells in 15 ml polypropylene centrifuge tubes. After a 30 minute incubation at room temperature the cells were washed with 2.0 ml of PBS and centrifuged for five minutes at 500 x g. Supernatants were separated from the cell pellets, both were transferred to 12 x 75 counting tubes, counted in a gamma well counter (Gamma 4000, Beckman Inst., Irvine, CA) and the percentage of activity associated with the cell pellet was determined. Fifty three percent of the activity was found to be associated with the cell pellet. While the maximum possible value in this assay is about 80%, the degree of retention is dictated by a number of factors including the affinity of the molecule for the target antigen. These results are within the normal range observed for radioiodinated C6.5 scFv, indicating to us that the C6.5-Fc was still reactive with cell surface HER2/*neu*.

Pharmacokinetic Studies

Four month old inbred male C.B17/Icr- *scid* (scid) mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Two days later, pharmacokinetic studies were initiated. Twenty micrograms of ^{125}I-C6.5-Fc were administered to three cohorts of four mice by i.v. tail vein injection and to three cohorts of four mice by i.p. injection. Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model #2007, Canaberra, Meridian, CT). Blood samples (20-75 uL) were obtained by retro-orbital bleeds from all mice at five minutes post injection and then from alternating groups at subsequent time points (e.g., group I at 15 minutes, group II at 30 minutes, group III at one hour, etc.). The blood samples were counted along with standards in a gamma counter (Beckman, 4000) and the percent of the injected dose retained per mL (% ID/mL) of blood over time was determined for each mouse. The mean values were determined for each time point and the pharmacokinetics were determined using the NCOMP program(57).

2.3.3 Construction of vector pPIgG-1

We have constructed a plasmid for the expression of single-chain antibody Fv domains (scFv) fused to the human IgG1 Fc domain to make a bivalent, ~106,000 molecular weight scFv-Fc fusion (Figure 2). Plasmid pPIgG1 for the expression of the scFv-Fc fusions (comprising the scFv followed by the hinge, C_H2 and C_H3 domains of human IgG1) is based on the *Pichia pastoris* expression plasmid pPICZαA (Invitrogen). This plasmid uses the alcohol oxidase promoter (AOX1) for high level expression of heterologous proteins, which are secreted to the media under direction of the yeast α-factor signal sequence. The human hinge, C_H2, and C_H3 domains of IgG1 were cloned into the *Not*I and *Xba*I sites of pPICZαA, creating plasmid pPIgG1. The presence of a stop codon at the end of C_H3 results in the myc epitope tag and $(His)_6$ sequences from pPICzαA not being expressed in the fusion protein.

scFv genes to be expressed as scFv-Fc fusions in pPIgG1 are amplified by PCR and subcloned into pPIgG1 using the 5' *Xho*I site in the alpha factor signal sequence and the *Not*I site at the end of the multiple cloning site. The 5' PCR primer, in addition to appending a dangling *Xho*I site, also recreates the amino acid sequence of the alpha amylase signal from the *Xho*I site to the end of the signal peptide, positioning the 5' end of the scFv flush with the end of the signal. The 3' PCR primer can be either a sequence-specific primer which appends a *Not*I site onto the end of the scFv gene, or a universal downstream primer for vectors such as pHEN1 which already have a *Not*I site positioned at the end of the scFv gene (58). The Fc domain adds an additional 26,300 in molecular weight to the scFv polypeptide chain, for a calculated molecular weight of ~53,000 for a typical scFv-Fc polypeptide monomer. As the selections for anti-uPA and anti-uPA-R single chain antibodies were ongoing at the time of the pPIgG-1 vector development, we chose two scFv as models to test the scFv-fusion vector's performance: C25, which recognizes the botulinum neurotoxin serotype A binding domain Hc (52), and C6.5, which recognizes the c-erbB-2 protein(53). The constructs were electroporated into Pichia pastoris strain GS115 for expression.

2.3.4 Expression and characterization of scFv-Fc fusions

Small scale expression and screening of Pichia transformants

Since expression from integrated pPICZαA-derived plasmids can vary depending on site of integration and copy number, multiple transformants were screened for expression level. Six clones apiece of the pPIgG1-C25 and pPIgG-C6.5 transformants (along with pPICZαA control) were grown in 10 mls of BMMY media at 30° C for 72 hours as described in Materials and Methods. Results for four clones of each scFv-Fc fusion are shown in Figure 3. By silver stain of the media samples (Panel A) under reducing conditions, a new band appears in the media that is not in the vector control, with an apparent molecular weight of ~70,000. Close inspection shows that there is actually a dimer of new material at this molecular weight.

Western blotting analysis (panel B) shows that these bands react with an anti-human Fc specific antibody. No anti-Fc reactive material was seen in control cells transfected with the control plasmid. Under non-reducing conditions, this material migrates in SDS-PAGE with approximately twice the apparent molecular weight, indicating that the scFv-Fc fusion secreted to the media is primarily in the form of a disulfide-linked dimer (not shown). Expression of the C6.5-Fc clones is somewhat less than for the C25-Fc clones. Expression was first observed at 24 hours with best expression levels seen at 72 hours (not shown). Two well-expressing clones were chosen for large scale expression and analysis.

Large scale expression and purification of scFv-Fc fusions

One liter of culture media was use used to generate pure C25-Fc and C6.5-Fc fusions for characterization. Briefly, the purification employed was ammonium sulfate precipitation of the proteins, followed by dialysis, Protein G chromatography, and gel filtration. Figure 4, panel A shows fractions from a typical purification. After ammonium sulfate precipitation, the fusion protein band is clearly visible, appearing as a dimer at ~70,000 daltons (lanes 1 and 4). After Protein G chromatography (lanes 2 and 5) the material is substantially pure, with a number of smaller bands copurifying which represent breakdown products which also bind to Protein G and react with anti-Fc antibodies (not shown). These breakdown species are removed by gel filtration on S200 (lanes 3 and 6). Under non-reducing conditions, the material migrates with approximately twice the molecular weight, indicating that the purified scFv-Fc fusions are disulfide-linked dimers (Figure 4B). The yield after purification was ~2 mg per liter of culture for C25-Fc and ~300 ug per liter of culture for the C6.5-Fc fusion.

The final purified material migrates in reducing SDS-PAGE as a tight dimer with an apparent molecular weight of ~70,000 daltons. This is substantially larger than the predicted molecular weights of 53,000 daltons for the reduced C25-Fc and C6.5-Fc fusion monomers. The discrepancy in apparent molecular weight and the heterogeneity observed could be due to glycosylation and/or differential processing of the alpha amylase signal peptide. To explore these possibilities, the purified fusions were subjected to N-terminal sequencing, de-glycosylation analysis with PNGase F, and mass spectrometry analysis.

N-terminal analysis results indicated that both the upper and lower bands of both of the fusions began with the amino acid sequence EAEA..., indicating that the signal peptide cleavage had occurred after the Kex2 site in the alpha amylase leader, but that Ste13 cleavage had not occurred.

The samples were further analyzed by deglycosylation with PNGase F (Figure 4C). After deglycosylation of both the fusion proteins, the doublet collapsed to a single band, indicating that the observed heterogeneity is due to difference in N-linked glycosylation.

Since SDS-PAGE can give spurious estimates of molecular weights, samples of the C25-Fc and C6.5-Fc were subjected to mass spectrometry analysis. The

molecular weights for the species were ~110,000 for both the C6.5-Fc and C25-Fc, in agreement with the predicted molecular weight from DNA sequence.

Pharmacokinetics

Single-chain Fv antibodies are rapidly cleared from the bloodstream in mouse models, with typical $t_{1/2}$ of the beta clearance of approximately 2.5 hours (42). This rapid clearance limits the usefulness of scFv in many potential animal models for efficacy where a longer serum half life is required. To test the pharmacokinetics of scFv-Fc fusions in mice, the C25-Fc fusion protein was radiolabelled and administered to scid mice in 20 ug doses, both intravenously and intraperitoneally. Figure 5 shows that the C25-Fc fusion had dramatically prolonged serum perseverance, with a $t_{1/2}$ for the beta phase 93 hours. The increased retention of the scFv-Fc fusions can be attributed to the increased size of the scFv-Fc homodimer which places it well above the renal threshold for clearance.

2.3.5 Discussion

Single chain Fv antibody fragments (scFv) are useful molecules for the design and construction of phage display libraries. This format is easy to construct in a library format and allows for monovalent display and selection in a single gene format. For these reasons the scFv is the format of choice for phage display antibody efforts.

Once an initial scFv is selected, however, this structure has a number of potential limitations for various types of characterization. The monovalent nature of the scFv makes it unable to partake in the additional binding interactions due to avidity as seen in a bivalent antibody. The limitation of off rate imposed by an scFv can limit the effectiveness of these molecules in many immunochemical applications such as FACS, ELISA, etc. The commonly utilized epitope tags for detection (Myc, E, FLAG) can be subject to proteolytic removal either during or after purification (Marks *et al.* unpublished observations). Also, in-vivo characterization of scFv's in animal models is limited by their rapid clearance from the bloodstream due to their small size. (42)

For many applications of characterizing a scFv it would be desirable to have a more "IgG-like" structure that combines the affinity and specificity of the scFv with the bivalency, pharmacokinetics, and effector functions of a complete immunoglobulin. One way is to clone the V_H and V_L genes from the scFv and reclone them into a full-length IgG expressing vector. However this can be laborious and time-consuming, especially if many such constructs have to be made and tested.

We have designed an intermediate construct, an scFv-Fc fusion in which the scFv is fused to the hinge, C_H2, and C_H3 domains of human IgG1, and shown that the fusion can be expressed and secreted as a glycosylated, disulfide-linked dimer in *Pichia pastoris*. The scFv domain of the fusion retains the affinity and specificity of the parent scFv, while the Fc region is recognized by Protein A, protein G, and anti-Fc antibodies, and is capable of directing antibody-dependent cellular cytotoxicity

(ADCC). Yields of the scFv-Fc fusions vary depending on the nature of the scFv, from ~2 mg/l for the best expressor (C25-Fc) to ~300 ug/l for the C6.5-Fc fusion in shaker flasks.

For the proposed *in vivo* anti-metastasis assays of scFv species, a long half-life in the serum will be needed. Previous experiments have shown that the $t_{1/2}$ for the β elimination phase for scFvs are typically 3.5 hours (42). In these studies we have demonstrated a $t_{1/2}$ beta 93 hours in a scid mouse for an scFv-Fc fusion, an approximately 25-fold improvement.

We are currently modifying the pPIgG1 vector system to increase their utility for anti-metastatic scFv characterization. At present cloning into this vector requires a PCR step to clone into the alpha factor signal sequence's *Xho*I site and reconstruction of the end of the signal flush with the start of the fusion. Since our N-terminal analysis results show that the final four amino acids (EAEA) are not cleaved from the fusion anyway, we are modifying the signal sequence to end in EAMA, incorporating a *Nco*I site, for full compatibility with the *Nco*I site in the pHEN1 scFv-phage display vector(58). We have constructed and are presently testing this vector (pPIgG2).

Glycosylation patterns on immunoglobulin species can have profound effects on antibody effector function and pharmacokinetics (reviewed in (59)). The serum retention observed for the scFv fusions may be affected by the type and extent of N-linked glycosylation. Yeast in general attach high-mannose residues (60), although in Pichia pastoris these residues are generally shorter than in Saccharomyces ceriviciae(61). Terminally mannosylated carbohydrates on immunoglobulins can be rapidly cleared by binding to high-affinity mannose receptors in the liver; this clearance can be partially prevented by co-injection of mannan (62). Since complete deglycosylation of an IgG can still yield a species with normal antigen affinity and retention of some but not all effector functions(59, 63, 64, 65), we are constructing a further refinement to these vectors (pPIgG3) in which will delete the N-linked glycosylation site from the Fc moiety. Hopefully this will lead to further increases in serum half life above that seen for the glycosylated scFv-Fc. This should result in fewer injections and lower doses of candidate scFv-Fc fusions for *in vivo* testing for anti-metastatic activity.

3. CONCLUSIONS

Metastasis - the uncontrollable spread of tumor cells throughout a breast cancer patient's body - is all too often the ultimate cause of the breast cancer patient's death. We seek to understand the molecular details of the metastatic process by developing high affinity, highly specificity antibody reagents to known or suspected molecules in metastasis and testing these antibodies in a variety of *in vitro* and *in vivo* assays for their ability to inhibit metastasis. Inhibition of metastasis is of critical importance to the survival of breast cancer patients. The proposed research will directly help to further understanding of metastasis, and could eventually lead to novel therapeutic approaches to the clinical prevention of metastasis. Previous studies have demonstrated a key role for the urokinase/urokinase receptor system in tumor penetration of basement membranes, and animal studies have shown that inhibition of receptor-bound uPA activity can inhibit metastasis. However, in all of these studies, some level of residual metastasis remains. For patient treatment, of course, the goal must be zero metastasis. This may be achievable not by any single intervention but by combination therapy directed towards two or more steps in the metastatic cascade. This proposal will establish general methods for determining the contribution of each step in breast cancer metastasis to the overall metastatic phenotype, in order to delineate the steps where therapeutic intervention would be most effective. In addition, the scFv species created herein could themselves be first-generation anti-metastatic agents for use in patients.

Toward this end we have selected six candidate antibody scFv fragments to two well known players in the metastatic process, urokinase-type plasminogen activator (u-PA) and its cell surface receptor (u-PA-R). These single chain antibodies were selected from a 7×10^9 member phage display antibody library created in the laboratory of Dr. James Marks. The antibody fragments have been sequenced and shown to be highly specific for the target antigens in ELISA assays. In addition, we have by competition ELISA shown that 5 of the 6 anti-uPA-R single chain antibodies compete with uPA for binding to the receptor uPA-R. These 5 antibody fragments are strong candidates for antibodies that will inhibit metastasis by disrupting the uPA:uPA-R binding interaction.

Phage display technology has made possible the direct isolation of these monovalent single chain Fv antibody fragments to urokinase and its receptor. For *in vivo* metastasis assays, however, it will be necessary to restore a long serum half life to these species, more reminiscent of a mature IgG molecule. We have constructed a vector for the simple, rapid expression of a single-chain antibody Fv domain (scFv) fused with the constant regions (hinge-C_H2-C_H3) of human IgG1 in the methylotrophic yeast *Pichia pastoris*. The scFv-Fc fusion protein is secreted and recovered in high yields from the culture medium as a covalent homodimer. These scFv-Fc fusions have a number of properties which make them useful tools for the characterization of scFv isolated from phage display libraries. The increased size of the glycosylated dimer (~106 kD vs. ~25 kD for a scFv) leads to prolonged serum

levels *in vivo*, with $t_{1/2}$ β increasing from ~2.5 hours for a typical scFv to 93 hours for a scFv-Fc fusion in mice. This property will be of benefit for studies where a longer serum half life is necessary to demonstrate an anti-metastatic effect. In addition, the human Fc portion of the fusion can be used to direct antibody-dependent cellular cytotoxicity (ADCC) to a target cell using human PBMCs as effectors. The bivalent nature of the scFv-Fc fusion leads to an increase in apparent affinity over that of the scFv alone due to avidity, leading to superior sensitivity in fluorescence activated cell sorting (FACS). Finally the Fc domain makes for a convenient, robust affinity handle for purification and immunochemical applications, bypassing the need for proteolytically sensitive epitope and/or affinity tags on the scFv.

We now have the tools in hand to continue to select scFv from phage display libraries that bind to known and suspected molecules in metastasis, and to convert these molecules into a format for *in vivo* assays of anti-metastasis effectiveness. In addition the scFv-Fc fusion technology developed is now being used to support studies of other antibody fragments in the laboratory of Dr. James Marks that may be used to ultimately treat breast cancer by interfering with growth factor receptors or angiogenesis factors.

The work presented in this report summarizes the completion of the following technical goals as outlined in the original research proposal:

1.1 Production of scFv against urokinase-type plasminogen activator. (months 1-6)
2.5 Production of scFv against uPA-R. (months 1-6)
2.6 Determination which of these scFv block uPA binding to uPA-R. (months 6-9)

In addition, technical objective 3.11:

3.11 Derivatization of scFv for prolonged serum half life and preparation of sufficient material for in vivo testing. (months 24-30)

Has been fast tracked to months 1-12 of the time line and largely completed. We will now proceed with the *in vitro* and *in vivo* characterization of the selected scFv against uPA and uPA-R.

4. FIGURES AND TABLES

ANTIGEN	ELUTED PHAGE TITER		
	round 1	**round 2**	**round 3**
urokinase (uPA)	3×10^4	10^7	10^7
urokinase receptor (uPA-R)	5×10^3	5×10^5	2×10^7

Table 1. Titers of eluted phage particles were determined for each round of selection by infecting a culture of E. coli TG1 cells with a dilution of the eluted phage, then plating to determine number of ampicillin resistant colonies.

ANTIGEN	ELISA POSITIVES		
	round 1	**round 2**	**round 3**
urokinase	3/96	12/96	22/96
urokinase receptor	0/96	18/96	28/96

Table 2. 96 clones from each round of selection were assayed by ELISA on the relevant antigen to determine the number of antigen-specific clones. No specific binders were detected for 96 random clones from the unselected library for either antigen (data not shown). A total of 37 total clones were detected for urokinase, and 46 for the urokinase receptor.

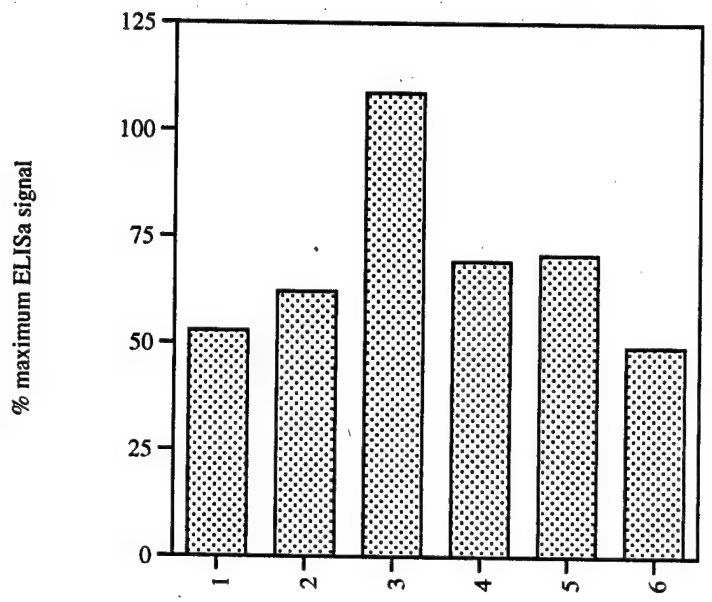

Figure 1. Competition ELISA to map if epitopes recognized by anti-uPA-R scFv clones overlap with uPA binding site on uPA-R. Results are expressed as percentage of maximal signal (in absence of uPA competition) that remains in presence of 5 ug uPA per well. Clone identification is as follows: 1, C2; 2, A4, 3, B5; 4, F5; 5, A9; 6, G12.

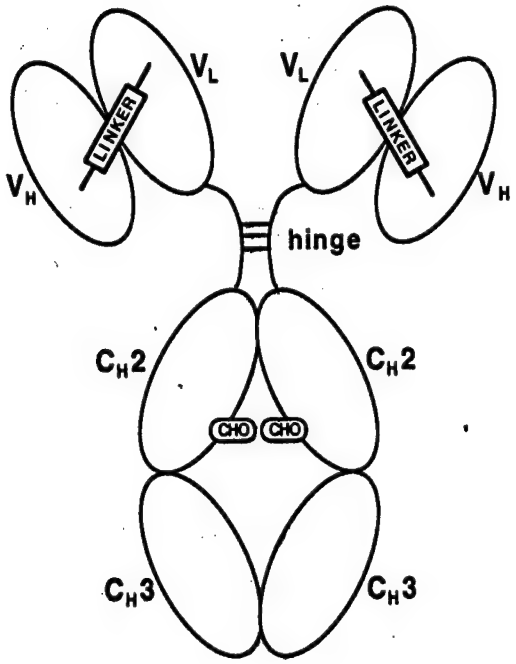

Figure 2. Schematic representation of an scFv-Fc fusion. scFv, single-chain Fv domain; V_H, heavy chain variable domain; V_L, light chain variable domain: C_H, heavy chain constant domain; -SS- indicates disulfide bonds between the hinge domains; -CHO, site of N-linked glycosylation.

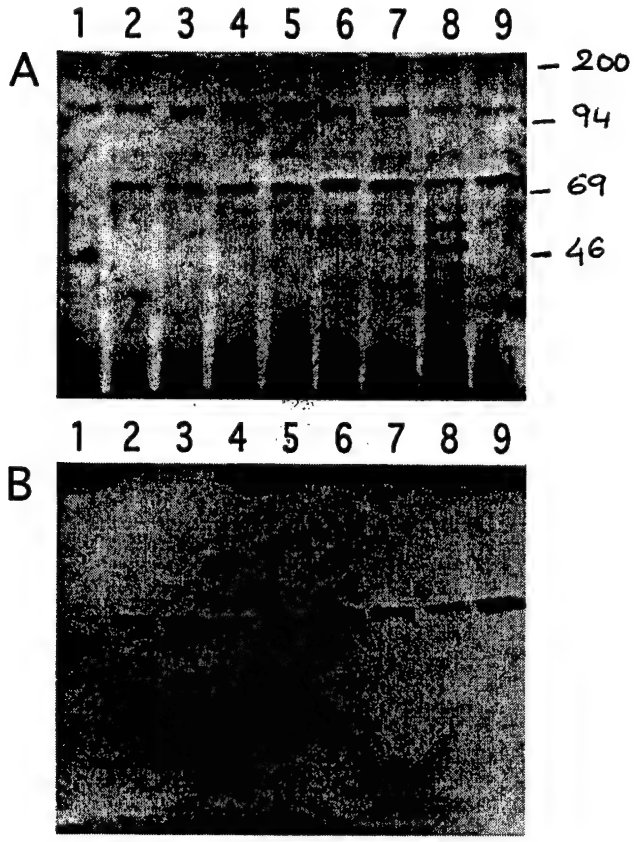

Figure 3. Small scale expression analysis of scFv-Fc expressing Pichia transformants. A) Silver stained SDS-PAGE gel. Lane 1, pPICZaA control transformant; lanes 2-5, pPIgG-C6.5 transformants; lanes 6-9, pPIgG-C25 transformants. B) Western blot of the above samples with anti-human IgG (Fc-specific) - horse radish peroxidase conjugates, followed by ECL development.

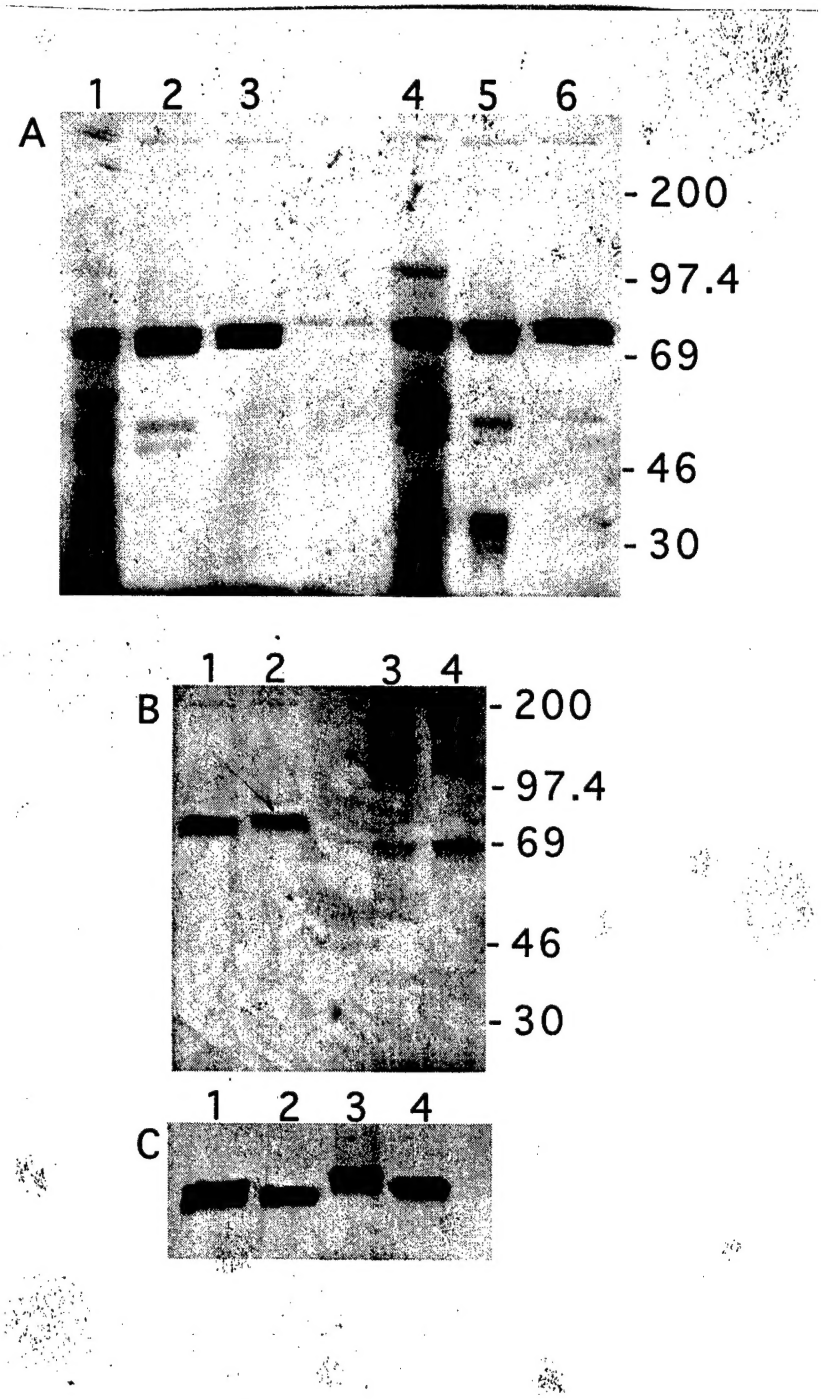

Figure 4. A) Samples from purification of the scFv-Fc fusions. Molecular weight standards are indicated with arrows. Lane 1, ammonium sulfate pellet of C6.5-Fc; lane 2, protein G pool of C6.5-Fc fusion; lane 3, S200 pool of C6.5-Fc. Lane 4, ammonium sulfate pellet of C25-Fc; lane 5, protein G pool of C25-Fc; lane 6, S200 pool of C25-Fc. B) Analysis of scFv fusions under reducing (lanes 1 and 2) and non-reducing (lanes 3 and 4) conditions. Lanes 1 and 3, C6.5-Fc fusion; lanes 2 and 4, C25-Fc fusion. C) Glycosylation analysis of scFv-Fc fusions. Lane 1, C6.5-Fc; lane 2, C6.5-Fc after PNGase F treatment; lane3, C25-Fc; lane 4, C25-Fc after PNGase F treatment.

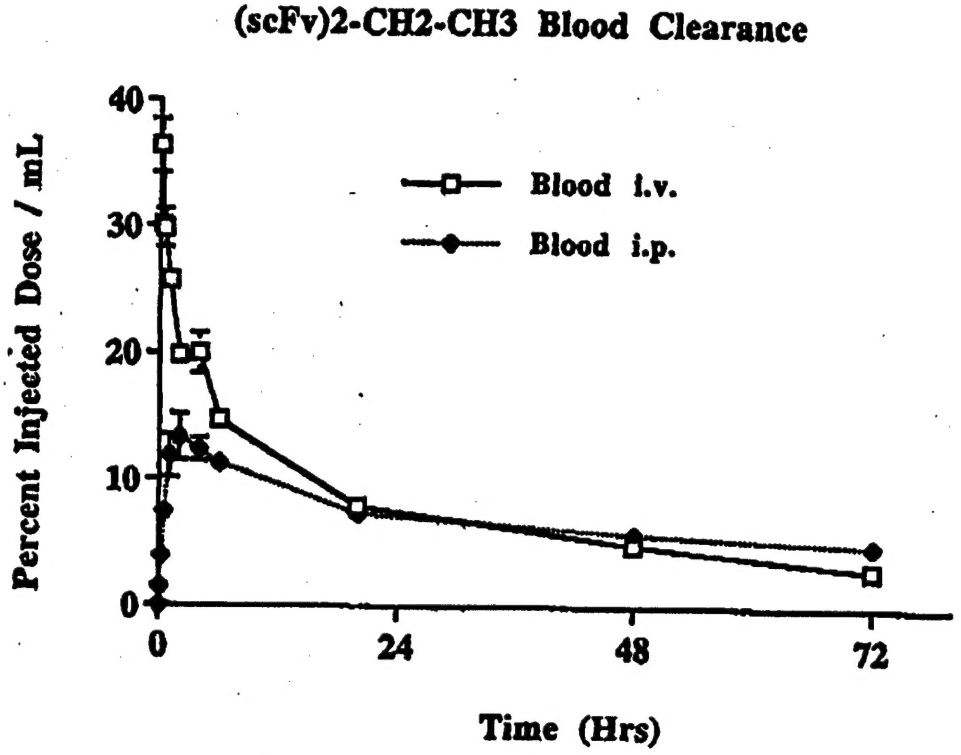

Figure 5. Pharmacokinetics of radiolabelled, model scFv-Fc fusion C6-5-Fc.

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